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Report

Paleogenomic sex inference of mammoth remains sheds light on the anthropogenic nature of bone accumulations

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SUMMARY

Whether large accumulations of woolly mammoth (*Mammuthus primigenius*) bones reflect natural mortality or deliberate human resource exploitation has long been debated, with major implications for understanding Late Pleistocene human-megafaunal interactions.[1–5] Here, we use ancient DNA to investigate site-formation hypotheses by comparing genetic sex ratios from mammoth remains recovered in putative anthropogenic bone accumulations and from geographically dispersed, non-anthropogenic contexts. We studied genome-wide data from 521 woolly mammoths—including 100 mammoths from bone accumulation sites and 421 mammoths from natural depositional settings across Eurasia and North America—of which 448 are newly generated. Genetic sex determination reveals a striking contrast between contexts: mammoths from dispersed sites show a male bias (~66.5%), which is consistent with the heightened vulnerability of solitary males to hazards such as natural traps, where bones are more likely to be preserved, whereas mammoths from anthropogenic bone accumulations are predominantly female (~70%). This female bias is pervasive across multiple sites, indicating an anthropogenic origin for these accumulations as a result of Upper Paleolithic hunters preferentially exploiting female mammoths, possibly derived from herd contexts. Together, these results provide population-scale genetic evidence that highlights the central role of mammoths in the subsistence and material economies of some Paleolithic communities.

RESULTS

Large faunal accumulation sites, formed through the deposition of bones from tens (and sometimes hundreds) of mammoths (genus *Mammuthus*), have been identified across Eurasia.[1–22] The origin of these accumulations can have multiple explanations; while some are the result of naturally occurring hazards (such as the La Brea tar pits),[23–27] others may have an anthropogenic origin (i.e., hunting and/or scavenging by humans).[1–21] Zooarchaeological research has

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identified extensive bone modifications, including cut marks, fresh-bone fracturing indicative of percussion damage, and occasional instances of stone points embedded in the bones at several Upper Paleolithic accumulation sites.[8,25,28–30] The anthropogenic nature of these sites is further supported by their spatial proximity to areas with signals of intense human

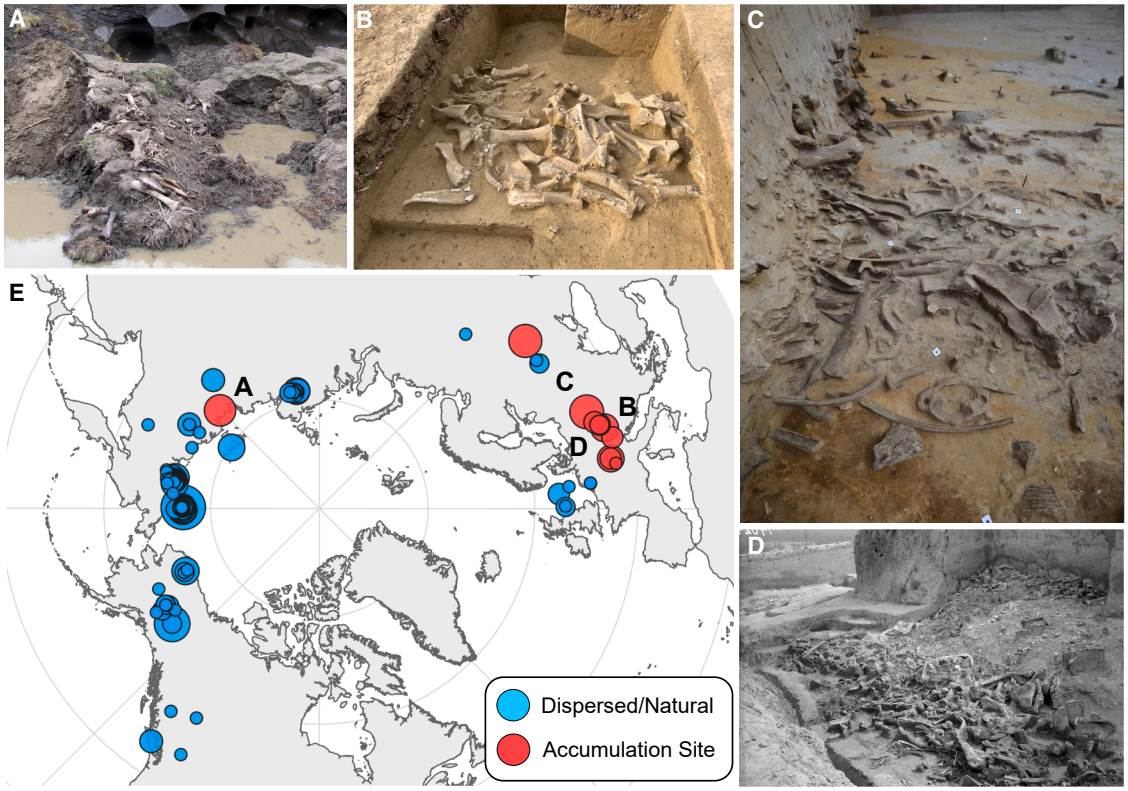

Figure 1. Excavation documentation for four bone accumulation sites and the geographical locations of the woolly mammoths analyzed in this study

(A–D) Bone accumulations from the sites of (A) Mus-Khaya, (B) Langmannersdorf, (C) Kraków Spadzista, and (D) Dolní Věstonice 1.

(E) The geographical locations of all analyzed woolly mammoths (n = 521, from 11 accumulation sites and 420 dispersed contexts). Red circles indicate sites with putative anthropogenic provenance, and blue circles represent natural depositional contexts. Circle size is relative to the number of individual specimens sampled per location. The map uses a Lambert azimuthal equal-area projection centered on the North Pole and was generated using the *Natural Earth* dataset. See Data S1A for detailed sample metadata. Photo credits: (A) Albert Protopopov, (B) Marc Händel, (C) Jarosław Wilczyński, and (D) Institute of Archaeology of the Czech Academy of Sciences, Brno.

activity, including hearths and anthropogenic occupational layers. Isotopic data from the remains of humans that were present in ecosystems that included mammoths have also revealed their importance in the human diet.[31,32]

In a previous genomic study of dispersed woolly mammoth remains (i.e., geographically isolated bone/tusk finds), a significant bias in sex ratios was reported, with 69% of the individuals being identified as male.[33] Due to the dispersed contexts and lack of signs of hunting activity, they likely represent individuals that died through a variety of non-anthropogenic processes.[33] Similar trends have been observed for other mammal species, such as steppe bison (*Bison priscus*) and brown bears (*Ursus arctos*),[34] which suggests that this overrepresentation of males is driven by a higher male exposure to risks such as wider dispersal ranges.[33–35] Contrasting with this, Rey-Iglesia et al.[36] found a predominance of females (57%) among the mammoth bones from a human-built circular bone structure at Kostenki 11-Ia using a combination of aDNA and proteomic sex determination. Radiocarbon dating showed a range of ages across individuals, which supported repeated human resource use of mammoths through time rather than a single mortality event. These localized signals prompt a broader question of whether comparable sex- and age-structured exploitation patterns characterize mammoth accumulations at other sites of putative human origin. To better understand the nature of woolly mammoth bone accumulation sites and the insights they can provide into human exploitation strategies, we analyzed the genetic sex of 100 woolly mammoths (62 of which are newly sequenced) found in bone accumulation sites of putative anthropogenic origin (i.e., possible human exploitation, henceforth "accumulations") and 421 (386 of which are newly sequenced, including genome-wide data from individuals previously published in Pečnerová et al.)[33] found in non-anthropogenic settings (i.e., natural settings, henceforth "dispersed") from across Eurasia and North America (Figure 1).

For genetic sex inference, we used an approach that accounts for the number of reads mapping to the X and Y chromosomes as compared to those mapping to the autosomes[37,38] (see further discussion in the STAR Methods section). Relying on a minimum of 4,000 mapping reads per individual, we confidently determined the genetic sex of all 521 woolly mammoths in our dataset (Figure 2; Data S1A). We find that 310 of them are genetically male (XY), 210 are genetically female (XX), and one individual exhibits a sex chromosome aneuploidy. We did not include any dispersed remains that have any reported association with anthropogenic activity, but a fraction of them could have

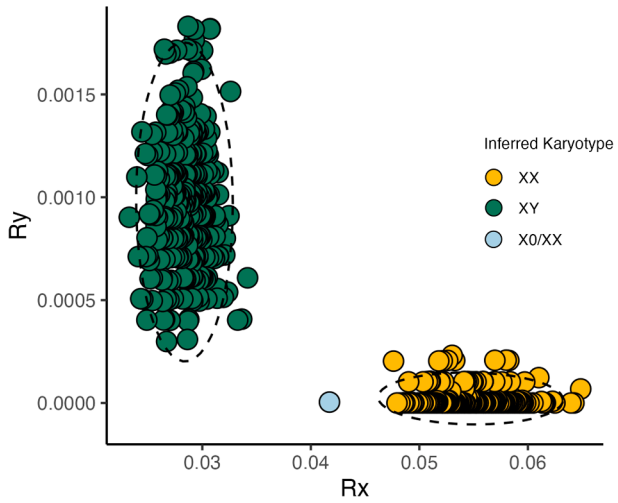

Figure 2. Genetic sex determination for all 521 woolly mammoths in this study

Genetic sex inference based on normalized read counts of sex chromosomes relative to the autosomes, displayed as Ry (ratio of reads mapping to Y chromosome to autosomal mapped reads) to Rx (ratio of reads mapping to X chromosome to autosomal mapped reads). Individuals are colored by inferred genetic sex. One individual with a possible sex chromosome aneuploidy is shown in light blue. Ellipses represent two standard deviations from the mean, using the stat_ellipse() function of the ggplot2 R package.

See also Figures S1–S4 and Data S1.

originated as a consequence of human activity. Among individuals from dispersed natural sites (n = 421), we identified 140 females (33.3%), 280 males (66.5%), and one sex chromosome aneuploidy (Figures 2 and 3). To assess whether the observed sex-ratio pattern was consistent across the geographical distribution of samples, we subdivided the samples by region: North America, Europe, and Siberia. Across all regional subsets, the male overrepresentation remained pronounced (Figure S3). We also investigated whether tissue type (molar, tusk, bone, or soft tissue) was driving the genetic sex ratios due to differential preservation of tissue types or different paleontological collection practices and found no significant differences (Fisher's exact test, p value 0.5741, Figure S2). Taken together, these findings indicate that the predominance of males in dispersed contexts is a widespread, stable feature of woolly mammoth specimens, rather than an artifact of sampling or regional preservation bias.

In contrast, we found that woolly mammoth remains were predominantly female (70 out of 100 individuals, 70%) across the analyzed bone accumulation sites of putative anthropogenic origin. To examine whether this pattern was consistent across sites, we compared sex ratios at each accumulation site individually (Figure 3). At Kraków Spadzista (the accumulation site with the highest number of individuals represented, n = 26), we observe a significant female bias (p = 0.009355). Furthermore, all accumulation sites represented by more than five individuals in the study exhibited a female bias (Data S1C). The contrast in sex ratios between dispersed sites (assumed to be primarily naturally formed) and bone accumulation sites is therefore likely to reflect differences in how these assemblages formed.

We also pooled together sites by type (dispersed and accumulation) and observed that the sex ratio in each of these categories is statistically significantly different from the null hypothesis of 50% females and 50% males using a binomial test. This ratio was selected as the null hypothesis because this is the ratio of individuals born in most mammalian species (see STAR Methods for more details). Across the pooled dataset, a Fisher's exact test comparing accumulation and dispersed sites (matrix(c(140, 280, 70, 30), 2, 2)) yields a p value = 3.37×10^{-11}, which confirms a strong and consistent pattern of female-biased accumulations versus male-biased dispersed sites (see STAR Methods).

One of the individuals in our dataset, MD067, had Ry and Rx values consistent with an X0/XX karyotype. This genome was generated from a long bone fragment collected on Wrangel Island (see Figure S4). To further investigate this, we examined the relative depth of coverage per chromosome in this sample and compared this to two individuals with inferred XY (bcm019) and XX (hmm023) karyotypes (Figure S1). We find that the proportion of reads from MD067 mapping to the X chromosome is likely a consequence of mosaicism, with around half of the cells carrying an X0 karyotype (55,X0) and the remainder carrying a 56,XX karyotype. XX/X0 mosaicism is well-documented in modern humans and inferred by intermediate X-chromosome depth of coverage between complete X0 and XX individuals, as reported in large-scale datasets such as the UK Biobank.[39] Recent studies conducted in ancient humans have identified the presence of individuals with autosomal and sex chromosome aneuploidies, including the 45,X0/46,XX mosaic karyotype.[37,40–44] Given the fragmentary nature of the bone, we could not examine it for any morphological features that might be associated with this aneuploidy. To our knowledge, this is the first report of such a condition in an extinct species, and we expect that as faunal ancient DNA studies continue to scale up sample sizes and implement sex inference pipelines that can infer karyotypes beyond XX and XY, so the identification and reporting of such sex chromosome aneuploidies will become increasingly common.

DISCUSSION

It has been hypothesized that male mammoths are more frequently recovered from natural death assemblages because, in polygynous species, males are expected to be more risk-taking.[45] This made them more likely to enter depositional settings, such as natural traps or areas subject to landslides, where bones are more likely to be preserved.[33,35] Thus, under a "natural-trap" model, mammoth accumulation sites would be expected to have a strongly male-biased sex ratio. If such accumulations instead represent alluvial deposits and are therefore only a consequence of overall mortality and taphonomic processes, then an even sex ratio would be expected. Consequently, our finding of a marked female bias in the woolly mammoth bone accumulation sites investigated here suggests another process for the formation of these sites, which is likely anthropogenic and involves the selective exploitation of female mammoths.

Material evidence from these accumulation sites strengthens this interpretation. At Kraków Spadzista, the presence of projectile points embedded in mammoth bones, spatially organized

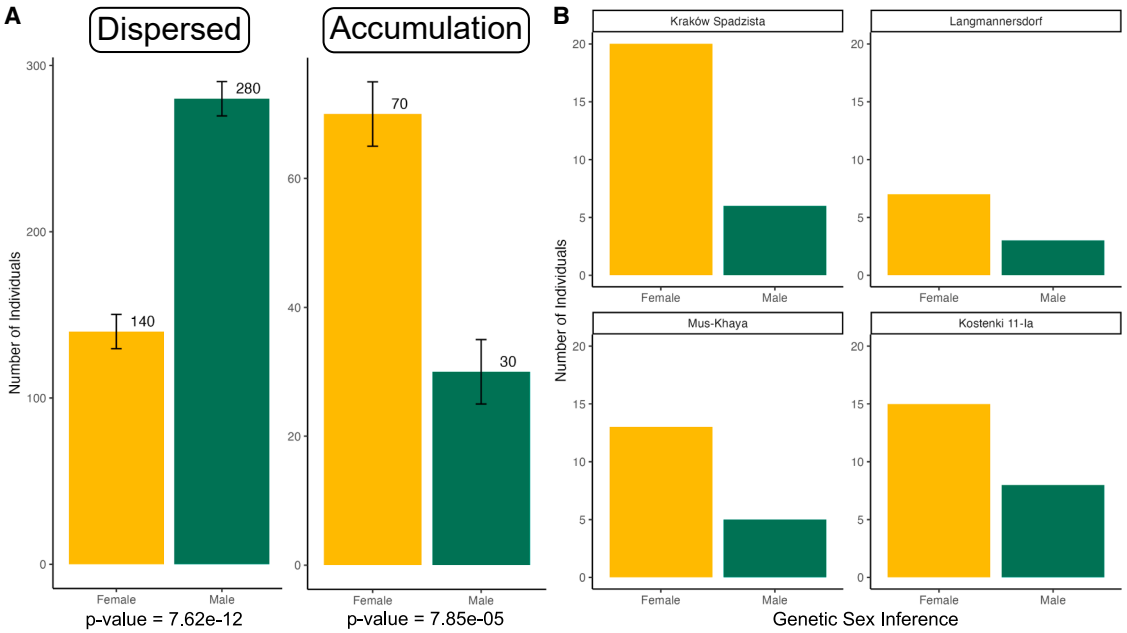

Figure 3. Genetic sex ratios for the different woolly mammoth contexts analyzed in this study

(A) Counts of female and male individuals identified in all samples from dispersed natural contexts and from all 11 accumulation sites grouped together. *p* values represent the results of a binomial test, in which the null hypothesis is a 50/50 female:male ratio, and error bars represent the standard error for the binomial test (for details, see STAR Methods).

(B) The total count of female and male individuals at all four bone accumulation sites with 10 or more individuals represented in the study.

See also Figure S3 and Data S1.

bone concentrations, and cut marks on remains all point toward active human involvement in mammoth processing (see supplemental information).[3,8,46] Anthropogenic indicators, such as impact marks and peri-mortem breaks, charred fragments, as well as possible cut marks, together with the presence of lithic artifacts, were also found in other accumulation sites analyzed in this study, such as Langmannersdorf.[47] At Kostenki 11-Ia, the predominance of females, the presence of all age classes, the spatial clustering of bones of different radiocarbon ages within the circular mammoth-bone structure, and occasional cut marks all indicate human exploitation rather than natural mortality events.[36] At Mus-Khaya, projectile point fragments embedded in mammoth scapulae indicate deliberate hunting practices.[25] Furthermore, studies of the mammoth-bone complexes at the Epigravettian Yudinovo site (Bryansk Oblast, Russia) indicate that these structures were built from body parts of recently deceased mammoths.[21] Additionally, the scarcity of carnivore tooth marks and gnawing traces in such large bone accumulations suggests human ability to control access to the carcasses during exploitation.[48]

Our genetic sexing results show that female mammoths are overrepresented in Paleolithic human-associated bone accumulations. While this pattern is consistent with the preferential targeting of females, it does not on its own demonstrate selective targeting of matriarchal herds and may also reflect other ecological, behavioral, or taphonomic processes. While it is possible that Ice Age humans avoided hunting adult males due to their size, females in matriarchal herds may not necessarily have been less dangerous, as group defense could have made encounters with herds equally or even more risky. One alternative explanation is that the observed female bias reflects how mammoths were encountered. Assuming that woolly mammoths lived in matriarchal herds composed primarily of females and their offspring, humans may have more frequently encountered and exploited these groups simply because herds are easier to predictably locate than solitary individuals.[49,50] In addition, females accompanied by calves may have been less mobile and therefore easier targets. This same predictability could also account for a female overrepresentation under scenarios of scavenging rather than active hunting. The more predictable movements of female-led herds may have made carcasses easier for hunter-gatherers to locate and repeatedly exploit. At the Yudinovo site, a female-biased sex ratio based on long-bone osteometrics and an age profile dominated by adult females was inferred, thus suggesting the selective exploitation of mother-offspring family units.[20,21] Future work integrating detailed age and genetic sex may make it possible to infer whether sites represent matriarchal herd contexts by testing whether male-biased mortality at natural sites is concentrated among older, dispersing males, which is consistent with suggestions that males leave matriarchal groups at around 13–15 years of age,[51,52] and whether female bias in anthropogenic accumulations varies by age class.

Modern elephant mortality studies show that carcass distributions are structured by ecological variables such as proximity to water, habitat quality/forest cover, proximity to human settlements, and events such as drought.[53–55] Additionally, ethnographic and historical accounts of elephant hunting provide useful observations on human-proboscidean interactions but are shaped by economic conditions that differ markedly from prehistoric subsistence contexts.[56] Integrating such insights

with age-stratified data and isotope analyses from accumulations of extinct proboscideans could, in future studies, offer additional insights into mammoth mortality patterns. At the same time, research on extinct proboscideans may offer a complementary perspective, thus helping to establish a comparative baseline for how present-day elephant populations might behave in landscapes less constrained by modern anthropogenic pressures (see further discussion in Methods S1).

Mammoths were a key resource for Upper Paleolithic hunter-gatherer societies across Eurasia, as they supplied meat and fat, ivory used to produce tools, portable art and ornaments, skeletal elements for the construction of mammoth-bone circular structures, and materials incorporated into burial contexts, such as ivory beads or mammoth scapulae placed with human skeletons.[57–62] Our results provide new insights into mammoth resource use and exploitation. By comparing genetic sex ratios across a large dataset of woolly mammoths from dispersed contexts and bone accumulation sites, we identify a consistent contrast between these assemblages. The predominance of males among dispersed individuals likely reflects natural mortality processes affecting solitary males, whereas the strong female overrepresentation observed at accumulation sites points to human involvement in their formation. While this pattern does not necessarily indicate deliberate targeting of matriarchal herds alone and may also reflect other encounter dynamics or scavenging opportunities, it nevertheless demonstrates that mammoth exploitation by Upper Paleolithic people was structured and recurrent. Together, these results provide population-scale genetic evidence that clarifies the origins of many mammoth bone accumulations and highlight the importance of mammoths as a central resource in the subsistence and material practices of Late Pleistocene hunter-gatherers.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Hannah M. Moots (hannah.moots@su.se).

Materials availability

This study did not generate any new, unique reagents.

Data and code availability

- Sequencing data (10,000 mapped, deduplicated reads for downsampled individuals, 4,000–10,000 mapped, deduplicated reads for all individuals with less than 10,000 deduplicated reads mapping to mEleMax1) from all newly generated woolly mammoth genomes have been deposited at the European Nucleotide Archive under the project number ENA: PRJEB122824 and are publicly available as of the date of publication. All accession numbers are listed in the key resources table.
- This paper does not report any original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

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DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE

SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
USER enzyme	New England Biolabs	NEB #M5508
AccuPrime reaction mix	Life Technologies	Cat #12344040
AccuPrime Pfx DNA polymerase	Life Technologies	Cat #12344024
EDTA	ThermoFisher Scientific	Cat #15575020
UREA	VWR	Cat #443874G
Proteinase K	VWR	Cat #1.24568.0100
Tango Buffer (10X)	ThermoFisher Scientific	Cat #BY5
ATP (100mM)	ThermoFisher Scientific	Cat #R0441
T4 Polynucleotide Kinase (10U/ul)	ThermoFisher Scientific	Cat #EK0032
T4 DNA Polymerase (5U/ul)	ThermoFisher Scientific	Cat #EP0062
T4 DNA Ligase (5U/ul)	ThermoFisher Scientific	Cat #EL0011
Bst Polymerase, LF (8U/ul)	New England Biolabs	Cat #M0275S
Maxima SYBR Green/ROX qPCR Master Mix (2X)	ThermoFisher Scientific	Cat #11873913
Agencourt AMPure XP beads	Beckman Coulter	Cat #10136224
Critical commercial assays		
MinElute purification columns	QIAGEN	Cat #28115
QiaQuick PCR purification kit	QIAGEN	Cat #28106
Agilent High Sensitivity kit	Agilent	Cat #5067-4626
Deposited data		
Raw sequencing data	This study	ENA: PRJEB122824
Raw sequencing data	Palkopoulou et al.[65]	ENA: ERP008929
Raw sequencing data	Fellows Yates et al.[66]	ENA: PRJEB21582
Raw sequencing data	Maschenko et al.[67]	ENA: PRJNA817536
Raw sequencing data	van der Valk et al.[68]	ENA: PRJEB42269
Raw sequencing data	van der Valk et al.[69]	ENA: PRJEB52742
Raw sequencing data	Díez-Del-Molino et al.[70]	ENA: PRJEB59491
Raw sequencing data	Dehasque et al.[71]	ENA: PRJEB52803
Raw sequencing data	Dehasque et al.[72]	ENA: PRJEB75909
Raw sequencing data	Rey-Iglesia et al.[36]	ENA: PRJNA897816
Software and algorithms		
DNA Harvester	Sharif et al.[73]	https://github.com/NBISweden/DNAharvester
BWA aln version 0.7.18-r1243	Li and Durbin[74]	https://bio-bwa.sourceforge.net/
SAMtools v1.17	Li et al.[75]	http://www.htslib.org/
fastp version 0.24.0	Chen et al.[76]	https://github.com/opengene/fastp
Fastqc v.0.11.9	Andrews[77]	https://packages.guix.gnu.org/packages/fastqc/0.11.9/
Mapdamage2 v.2.2.	Jónsson et al.[78]	https://ginolhac.github.io/mapDamage/

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Sampling and dataset

The mammoth specimens were obtained from multiple sources, including curated specimens from museum collections as well as newly recovered remains from recent archaeological and paleontological excavations, collected opportunistically, or identified during systematic surveys (Data S1A). We generated genomic data for 448 woolly mammoth samples, 62 from six bone accumulation sites, including Kraków Spadzista (Poland), Mus-Khaya (Siberia), Langmannersdorf (Austria), Dolní Věstonice

I (Czechia), Milovice I (Czechia), and Předmostí I (Czechia) (See Supplementary Materials for additional information on each site), and 386 from specimens collected at sites of natural setting in Europe, Siberia, and North America (Alaska and Yukon) (Data S1A; Figure 1). We additionally curated a panel of previously published genome-wide data from 73 woolly mammoths spanning the study period and geographic area.[36,65,66,68,70–72,79] The final dataset included genomic data from 521 woolly mammoths: 421 from natural sites, and 100 from bone accumulation sites (Figure 1; Data S1A). To minimize the possibility that multiple samples were derived from the same individual, specimen selection was guided by paleontological and archaeological context and anatomical criteria, as described in STAR Methods "sampling strategy and identification of unique individuals".

METHOD DETAILS

Sampling strategy and identification of unique individuals

To minimize the possibility that multiple samples derived from the same individual, specimen selection was guided by paleontological and archaeological context and anatomical criteria, as described below.

Dispersed sites

Dispersed finds of mammoth remains signify bones, teeth and tusks that have been randomly encountered during field work (i.e. are not associated with accumulations or human sites). These are typically found >100 metres apart, or from sections that span tens of thousands of years of time, and are thus unlikely to derive from the same individual.

Accumulation sites

In taphonomic and archaeozoological contexts, a bone accumulation is best understood as the outcome of processes that gather skeletal elements at a particular location. As defined by R. Lee Lyman, "a bone assemblage is the result of a series of processes, including the accumulation of skeletal elements by biological and geological agents," and more specifically, "accumulation refers to the processes responsible for the gathering of bones at a particular location".[80]

For archaeological accumulation sites, sampling focused preferentially on molars that have previously been assigned to individuals during zooarchaeological analysis, whenever possible. For example, at Kraków Spadzista, specimens were selected from individuals identified in prior zooarchaeological work (e.g., Haynes et al.[81] and Wojtal et al.[82]). All samples studied from Langmannersdorf, Milovice I - Sector G, Předmostí I, Dolní Věstonice are molars and all but one sample from Kraków Spadzista were molars (with the other sample being a petrous bone).

When teeth were preserved in situ within mandibles, only a single tooth was sampled from each specimen to avoid duplicate sampling. When isolated teeth were sampled, several anatomical and morphological criteria were used to minimize duplication. Mammoths typically have only two functional molars in occlusion per quadrant (e.g. left mandible, right mandible, left maxilla, right maxilla, totaling eight teeth in the mouth), except for brief periods during tooth replacement early in life.[83] Consequently, teeth that differ substantially in rank (e.g., dp3 vs. M3) must derive from different individuals. When teeth of the same rank were present, siding (left vs. right; upper vs. lower) was considered, since only one tooth of each rank occurs in a given quadrant, so we can also rule out that two M2 teeth from the same quadrant (e.g. lower left), they cannot possibly belong to the same individual.

Additional factors such as tooth size, morphology, and wear stage were also considered when selecting specimens, as teeth belonging to the same individual are generally similar in these characteristics.

For several archaeological assemblages included in this study, zooarchaeological analyses have previously estimated the minimum number of individuals (MNI) based on skeletal element representation, particularly molar counts and siding. In these assemblages, the estimated MNI substantially exceeds the number of specimens sampled for ancient DNA analysis. For example, MNI estimates derived from molar elements (considering tooth rank and sidedness) indicate that the number of individuals represented in these assemblages is considerably larger than the subset analyzed genetically. As a result, the sampling strategy represents a smaller number of individuals than the minimum number of individuals estimated to be present in the assemblages.

Together, these contextual and anatomical criteria were used to minimize the likelihood of sampling the same individual more than once.

Accumulation Site	Number of Individuals Studied	MNI (Minimum Number of Individuals)	Reference
Kraków Spadzista	26	113	Wojtal et al.[82]
Mus-Khaya	18	50	Plotnikov et al.[84]
Langmannersdorf	10	27	Bosch[85]
Milovice I - Sector G	3	21	Péan[86]
Předmostí I	3	>1000	Musil[87]
Dolní Věstonice I	2	17	Wilczyński et al.[88]

Minimum Number of Individuals for Sites with Newly-Reported Data. Table shows all sites with newly-reported genomic data in this study, the number of individuals studied, the MNI (Minimum Number of Individuals) estimated per site by zooarchaeological assessment, and the citation for each MNI reported.

Additional information about all accumulation sites with newly-reported data is provided in Methods S1.

Laboratory methods

DNA extraction and library preparation were carried out in the ancient DNA laboratory facilities at the Centre for Palaeogenetics in Stockholm (Sweden), the University of Potsdam (Germany), and the University of York (UK). In all cases, an ancient DNA standardized procedure was followed using protective suits, gloves, and face masks, as well as ultraviolet (UV) irradiation in hoods, entry rooms, and of tools to limit possible modern or cross-contamination. Negative controls were included during all laboratory steps.

DNA extraction

Extraction protocols for DNA extraction are specified per sample in Data S1B. ''Drilling'' indicates that bone powder from the sample material was collected using a hand-held Dremel drill. "Powdering", that the tusk or bone surface was first cleaned using a Dremel drill bit, after which a small fragment was removed and powdered with a pestle and mortar. ''Crushing'' indicates that samples were fragmented using a mortar and pestle. ''Bleach-treatment'' indicates that, before DNA extraction, a 0.05% bleach solution was applied for four minutes and followed by three washes with molecular-grade water. In all cases, DNA was extracted from between 20 - 50 mg of material following the Dabney silica-column-based protocol.[89]

Library preparation

Briefly, for most samples, we extracted DNA and built double-stranded DNA libraries.[89,90] Libraries were treated with Uracil-Specific Excision Reagent (USER) enzyme to remove post-mortem damage,[91] and Tween-20 was added to the elution buffer to increase DNA recovery.[92] For a subset of samples, we built single-stranded sequencing libraries following Gansauge and Meyer[93] with additional modifications,[94,95] including treatment of DNA extracts with uracil-DNA glycosylase (UDG) to remove uracils at non-terminal base positions resulting from cytosine deamination.[93–95] A subset of libraries was additionally processed using the single-stranded DNA library preparation protocol described by Kapp et al.[96] Additionally, some extractions and libraries were carried out using the 96-well plate clean-up protocol described in Gilardet et al.[92] Specifics on which samples were processed following each protocol can be found in Data S1B. In all cases, the optimal number of PCR cycles was estimated before indexing amplification using qPCR. DNA concentration in each library was quantified by measuring fragment size on a Tapestation (Agilent Technologies). Sequencing was performed on NextSeq, HiSeqX, and NovaSeq Illumina platforms.

Bioinformatic processing

Both newly sequenced and previously published samples (Data S1A) were processed using the same Nextflow-based DNA harvester pipeline.[73] First, raw reads were adapter-trimmed and merged using fastp version 0.24.0,[76] excluding those below 30bp in length. Merged reads were mapped against a concatenated reference genome of an Asian elephant (mEleMax1, https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_024166365.1/) plus a human genome (GRCh37/hg19) using BWA aln version 0.7.18-r1243[74] and commonly used ancient DNA settings (-l 1024 -n 0.01 -o 2).[76] The human reference was added as a decoy to prevent any reads originating from potential human contamination from mapping to the mEleMax1 genome.[97] BAM files were subsequently sorted and merged per sample, and PCR duplicates, human-aligned reads, as well as reads with a lower mapping quality of 25 (MQ25) were removed with samtools v1.17.[75] We used a minimum threshold of 4,000 mapping reads per individual for genetic analyses. To account for differences in depth of coverage across samples, for those samples with more than 10,000 reads mapping after filtering, we randomly sampled 10,000 mapping reads to perform genetic sexing. The dataset is composed of 8 genomes with between 4,000 and 10,000 reads, and 440 downsampled to 10,000 reads (Data S1A). We have also uploaded higher-coverage data for MD067 (inferred X0/XX aneuploidy), which is 1,369,889 reads mapping to mElemax1, using the pipeline and filters described here.

QUANTIFICATION AND STATISTICAL ANALYSIS

Genetic sex inference

For genetic sex determination, we used the approach described in Dehasque et al.,[72] which follows a modified version of Anastasiadou et al.[37] that was originally developed for use with human data. Previous work on DNA-based sex inference in mammoths has relied on relative read coverage for the X chromosome in relation to the autosomes,[27,33] whereas this method takes into account three points of information: reads mapping to the X chromosome (annotated NC_064846.1 in mEleMax1), reads mapping to the Y chromosome (NC_064847.1), and the total number of reads mapping to the autosomes. This approach allows for genetic sex inference by comparing the ratio of reads confidently mapped to each respective sex chromosome to the total mapped autosomal reads, in order to assess the depth of coverage of each sex chromosome in relation to the autosomal depth of coverage. Thus, Rx and Ry are reads respectively mapping to the X or Y chromosome, divided by reads mapping to the autosomes. Simulations have shown that inference of genetic sex is possible with as few as 4,000 reads.[27] In addition to genetic sex determination, this approach also enables the identification of potential sex chromosome aneuploidies (e.g., XXY, XYY, X0).

To provide further support for the X0/XX karyotype call for MD067, we computed a Z-score, calculated by subtracting the individual's value (in this case, reads mapping to the X chromosome for MD067) from the mean (average) of the dataset and then dividing by the standard deviation of all XX individuals (Figure 2). The Z-score for MD067 is -3.75, indicating that the individual falls well outside the range of XX variation and is therefore consistent with an X0/XX karyotype.

Statistical analyses

Null hypothesis

For all statistical sex ratio tests, the null hypothesis used here assumes a 50:50 ratio of females to males, consistent with the typical birth sex ratio observed in mammalian species.

Dispersed versus accumulation sites

Although dispersed and accumulation sites broadly originate from natural and anthropogenic processes, respectively, these distinctions are not always clear-cut. Large natural mortality events (e.g., drought- or disease-related deaths) can produce undispersed assemblages, while some dispersed sites may reflect hunting or scavenging events for which direct archaeological indicators have not been preserved. For this reason, we employ descriptive categories (''accumulations'' and ''dispersed'') rather than assigning strict causal interpretations, and we consider contextual evidence such as embedded projectile points, butchery marks, and other traces of human activity when discussing likely anthropogenic assemblages. Indeed, some dispersed remains resulting from hunting have been directly identified,[98] although we did not include any dispersed remains that have any reported association with anthropogenic activity, but a fraction of them could have originated as a consequence of human activity.

While genetic sex inference has not yet been conducted on any woolly mammoth accumulation sites thought to be naturally formed, a recent study of Columbian mammoth remains from an alluvial deposition site in the Basin of Mexico identified 18 females and 25 males.[27] The difference in numbers is not significantly different from a 50% female:50% male ratio.

Error bars

The error bars in Figure 3 indicate the standard deviation of the binomial test calculated as $\sqrt{(n*p*q)}$, where 'p' is the probability of one outcome (e.g., XX, probability 0.5), 'q' is the probability of the other outcome (e.g., XY, probability 0.5, same as 1-p), and 'n' is the number of experiments (i.e., number of individuals).